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Growth inhibition of a *Fusarium verticillioides* GUS strain in corn kernels of aflatoxin-resistant genotypes

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Abstract Two corn genotypes, GT-MAS:gk and MI82, resistant to *Aspergillus flavus* infection/aflatoxin contamination, were tested for their ability to limit growth of *Fusarium verticillioides*. An *F. verticillioides* strain was transformed with a β-glucuronidase (GUS) reporter gene (*uidA*) construct to facilitate fungal growth quantification and then inoculated onto endosperm-wounded and non-wounded kernels of the above-corn lines. To serve as a control, an *A. flavus* strain containing the same reporter gene construct was inoculated onto non-wounded kernels of GT-MAS:gk. Results showed that, as in a previous study, non-wounded GT-MAS:gk kernels supported less growth (six- to ten-fold) of *A. flavus* than did kernels of a susceptible control. Also, non-wounded kernels of GT-MAS:gk and MI82 supported less growth (two- to four-fold) of *F. verticillioides* than did susceptible kernels. Wounding, however, increased *F. verticillioides* infection of MI82, but not that of GT-MAS:gk. This is in contrast to a previous study of *A. flavus*, where wounding increased infection of GT-MAS:gk rather than MI82 kernels. Further study is needed to explain genotypic variation in the kernel response to *A. flavus* and *F. verticillioides* kernel infections. Also, the potential for aflatoxin-resistant corn lines to likewise inhibit growth of *F. verticillioides* needs to be confirmed in the field.

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Introduction

Fusarium verticillioides (synonym *F. moniliforme* Sheldon; teleomorph *Gibberella moniliformis* (Sawada) Ito, in Ito and Kimura), a common corn pathogen associated with ear rots (Koehler 1959), produces a series of toxins on corn including the fumonisins. Fumonisins B_1, B_2 and B_3 are the major fumonisins produced in nature, fumonisin B_1 being the most prevalent of these produced in corn and believed to be the most toxic of all the fumonisins (Thiel 1992). While fumonisin production occurs under field conditions, contamination in corn also may occur during storage (Bacon and Nelson 1994). Concentrations as high as 330 μg g^{-1} (ppm) have been reported on corn and corn products (Bacon and Nelson 1994). Fumonisins have been associated with a variety of animal toxicoses but, as yet, human association with toxicosis remains inconclusive (Shelby et al. 1994). Recently, based on the established toxicity of fumonisins, the U.S. Food and Drug Administration issued a draft industry guidance on fumonisin levels in human foods (2–4 μg total fumonisins g^{-1}) and animal feeds (5–100 μg total fumonisins g^{-1}, depending upon the species; Food and Drug Administration 2000). Control of fumonisin contamination of corn, therefore, has become a food safety research priority.

Aflatoxins, another significant class of carcinogenic mycotoxins produced by *Aspergillus flavus* Link:Fr and *A. parasiticus* Speare (references in Brown et al. 1999), have been widely investigated due to their key role in establishing the significance of mycotoxins in inducing animal diseases (Wyllie and Morehouse 1978). Control of preharvest aflatoxins has been high-priority research for almost 30 years (reviewed in Brown et al. 1999). Unlike aflatoxins, however, only a few strategies for controlling preharvest *F. verticillioides* infection/fumonsin contamination of corn are currently being pursued. These include biological control of fungal growth by a bacterium or by fumonisin non-producing strains of *F. verticillioides* (Bacon and Hinton 2000; Plattner et al. 2000), exploitation of host-produced defense compounds (DIMBOA;

Glenn and Bacon 2000) and chemical control of fungal growth (Yates et al. 2000). Since aflatoxigenic fungi and fumonisin-producing fungi both affect corn and are often found together in field-isolated corn kernels (Lillehoj and Zuber 1988), it would be economical and save both time and effort if strategies and technologies being developed to control contamination of aflatoxins could simultaneously affect the formation of *Fusarium* toxins.

One of the strategies for controlling aflatoxins in corn is enhancement of host resistance; and several corn lines with natural resistance to *A. flavus* infection/aflatoxin contamination have been identified (references in Brown et al. 1999). However, the poor genetic backgrounds of these lines make them unsuitable for immediate commercial use. Aflatoxin-resistant genotypes, therefore, form the cornerstone of breeding programs aimed at transferring resistance to more suitable germplasm (Campbell and White 1995) and also are being investigated to identify specific traits to serve as resistance markers in breeding programs (references in Brown et al. 1999).

In the present study, the authors sought to determine whether the resistance mechanisms operating in two established aflatoxin-resistant corn genotypes were broad-based (showing efficacy against other fungal diseases) or specific only against aflatoxigenic fungi. To do this, we genetically transformed an *F. verticillioides* strain with a β-glucuronidase (GUS) reporter gene construct for quantifying fungal growth in corn kernels. The usefulness of the GUS gene fusion system in quantifying fungal growth in corn seed has been established, as has a reliable GUS protocol for measuring GUS activity in seed that we used in the present investigation (Brown et al. 1995, 1997). Here, as a control, we also employed an *A. flavus* strain containing the same reporter gene construct, which was used earlier in this laboratory to establish natural aflatoxin-resistance in corn (references in Brown et al. 1999).

Materials and methods

Corn entries

Seeds of the resistant breeding population GT-MAS:gk (Brown et al. 1995)were obtained from the USDA-ARS Insect Biology and Population Management Laboratory in Tifton, Ga.. Seeds of the resistant inbred MI82 (Campbell and White 1995) were obtained from the Department of Crop Sciences of the University of Illinois. Pioneer Hybrid Brand 3154 (P3154) seeds, susceptible to aflatoxin formation (Guo at al. 1996), were obtained from Pioneer Hi Bred International.

Fungal transformation and strain growth conditions

An *F. verticillioides* strain (GAP 2-4) transformed with the *Escherichia coli uidA* gene linked to a β-tubulin gene promoter was used to quantify GUS activity in maize kernels. An *F. verticillioides* strain, FGSC 7600 (Fungal Genetics Stock Center, Kansas City, Kan.), was cotransformed with two plasmids. One plasmid, pGAP2, contained a reporter gene construct consisting of the *benA* gene promoter from *A. flavus* (Seip et al. 1990), the *uidA* gene from *E. coli* (Jefferson et al. 1987) and an *A. nidulans* gene terminator (May et al. 1987). The second plasmid pH1S was a transformation vector based on *E. coli* plasmid pBR322 and carried a bacterial ampicillin gene (Turgeon et al. 1987). This plasmid contained promoter 1 sequences from *Cochliobolus heterostrophus* and the hygromycin B gene from *E. coli* as a selectable marker. Cultures were grown at 24 °C in the dark on potato/dextrose agar supplemented with hygromycin B. Conidia from 7- to 10-day-old cultures suspended in deionized water served as inocula.

The strain of *A. flavus* (GAP 2-4) used in this investigation is a transformant containing the *E. coli* GUS gene linked to an *A. flavus* β-tubulin gene promoter. It has been fully described in other studies (Brown et al. 1995). Cultures were grown at 37 °C in the dark on potato-dextrose agar. Conidia from 4- to 7-day-old cultures suspended in deionized water served as inocula.

Kernel inoculations

In the first experiment, kernels of GT-MAS:gk and P3154 were surface-sterilized, dipped into a suspension of *A. flavus* or *F. verticillioides* conidia (4.0×10^6 conidia ml^{-1}) and then incubated, using the kernel-screening assay (KSA) at 24 °C for 10 days, to facilitate fungal infection (Brown et al. 1995; 1997). Each treatment was replicated seven times with each replicate containing three kernels. The experiment was performed twice.

In the second experiment, kernels of GT-MAS:gk, P3154 and MI82 were either treated by wounding them through the pericarp to the endosperm to a depth of 1 mm, or were left unwounded. Wounding was performed with a 26-gauge, 13-mm hypodermic needle (Becton Dickinson & Co., Rutherford, N.J.; Brown et al. 1995). All kernels tested were then surface-sterilized, inoculated with *F. verticillioides* and incubated (KSA) for 10 days as above. Each treatment was replicated seven times with each replicate containing three kernels; and the experiment was repeated.

Quantification of GUS activity

After incubation, kernels of both experiments were subjected to a protocol for fluorogenic GUS quantification (Jefferson 1987), described in detail in a previous study (Brown et al. 1997). For each enzyme reaction, 10–50 µl of crude extract were added to the assay buffer containing the substrate and reactions were stopped after 10 min or 20 min. Preliminary β-glucuronidase kinetic experiments were performed to identify the amount of crude extract to use in each test and to identify a time that fell within the linear portion of the reaction curve. GUS activity in samples was determined using a Gilford Fluoro IV spectrofluorimeter (Corning Laboratory Sciences Co., Oberlin, Ohio); excitation was at 360 nm and emission at 455 nm. GUS activity was normalized through protein determinations in crude extracts by the method of Sedmak and Grossberg (1977) and was expressed in nanomoles of methylumbelliferone produced per minute per milligram of protein.

Statistical analyses

Analyses of GUS activity data were performed with the Statistical Analysis Software System (SAS Institute, Cary, N.C.). Treatment replicates from each test were first subjected to analysis of variance, followed by mean comparisons of either GUS values, log transformations of these values, or square-root transformations of these values. Differences among treatment means were determined by the least significant difference test.

Results

A. flavus GUS activity was lower in both tests (six- and ten-fold, respectively) in GT-MAS:gk kernels than in

Table 1 β-Glucuronidase (GUS) activity (fungal growth) in corn kernels inoculated with *Aspergillus flavus* or *Fusarium verticillioides*. P3154 is susceptible to *A. flavus* growth/aflatoxin production while GT-MAS:gk is resistant. Test values represent averages of specific GUS activity (nmol methylumbelliferone produced min^{-1} mg^{-1} kernel protein) for seven replicates in each test. Values followed by the same letter are not significantly different (P=0.05), using the least significant difference test. Data for the two tests were kept separate, due to a significant interaction (P=0.05) between test and treatment variables; and data were log-transformed prior to analysis to equalize variances

Entries	Inoculum	GUS-Test 1	GUS-Test 2
P3154	*A. flavus*	10.0 a	3.4 a
GT-MAS:gk	*A. flavus*	1.0 c	0.6 bc
P3154	*F. verticillioides*	2.2 b	1.1 b
GT-MAS:gk	*F. verticillioides*	0.6 c	0.5 c

Table 2 GUS activity (fungal growth) in maize kernels inoculated with *F. verticillioides*. P3154 is susceptible to *A. flavus* growth/aflatoxin production while GT-MAS:gk and MI82 are resistant. Values represent averages of specific GUS activity (nmol methylumbelliferone produced min^{-1} mg^{-1} kernel protein) for 14 replicates in two separate tests. Values followed by the same letter are not significantly different (P=0.05), using the least significant difference test. Data were log-transformed prior to analysis to equalize variances

Entries	Treatment	GUS Activity
P3154	Endosperm-wounded	174.3 a
	Non-wounded	133.8 a
GT-MAS:gk	Endosperm-wounded	42.2 c
	Non-wounded	57.3 bc
MI82	Endosperm-wounded	82.0 b
	Non-wounded	40.1 c

kernels of the aflatoxin-susceptible line, P3154 (Table 1). *F. verticillioides* GUS activity also was significantly less in GT-MAS:gk kernels than in P3154 (two- to four-fold) (Tables 1, 2). *F. verticillioides* GUS expression was also less in kernels of the resistant line MI82 than in P3154 (three-fold; Table 2). Wounding MI82 kernels (Table 2) prior to infection by *F. verticillioides* increased GUS activity (two-fold), but wounding GT-MAS:gk kernels did not affect GUS levels.

Discussion

Our previous studies have shown that an antifungal trypsin inhibitor protein (TI), isolated from corn kernels, is associated with resistance in both GT-MAS:gk and MI82 (Chen et al. 1998). *A. flavus* and *F. verticillioides*, however, respond differently to this protein (Chen et al. 1999). While TI was efficacious against in vitro growth of *A. flavus*, *F. verticillioides* and several other phytopathogenic fungi, *F. verticillioides* hyphae and conidia were less sensitive to TI than were *A. flavus* hyphae and conidia (Chen et al. 1999). The IC_{50} (calculated concentration required to cause 50% inhibition of hyphal growth) after 12 hours of incubation for *A. flavus* was 33 μg ml^{-1}, while that for *F. verticillioides* ranged from 100 μg ml^{-1} (microconidia used) to 124 μg ml^{-1} (macroconidia used; Chen et al. 1999). Other studies also have highlighted differences between these two species in their response to antifungal proteins (DeLucca et al. 1997). To compare in vivo effects on the growth of these species in aflatoxin-resistant corn kernels, we used two methods established in this laboratory: the KSA for facilitating fungal infection of corn seed and a fluorogenic protocol for quantifying fungal GUS activity in corn seed (Brown et al. 1997).

Differences in infection levels between *A. flavus* and *F. verticillioides* in susceptible line P3154 were significant and dramatic, being three- to approximately five-fold higher with *A. flavus*. However, this was not unexpected, since this *A. flavus* strain grew at least three times faster in culture than did the *F. verticillioides* strain. In the present study, the aflatoxin-resistant corn population GT-MAS:gk demonstrated resistance to *A. flavus* infection similar to that observed earlier (Brown et al. 1995). Growth of *F. verticillioides* also was inhibited in kernels of this genotype and in the resistant line, MI82, compared to growth in the susceptible line, P3154.

Non-wounded kernel resistance in GT-MAS:gk has been partially attributed to pericarp cutin and wax layers (Guo et al. 1995). The expression of wounded-kernel resistance in GT-MAS:gk and other resistant genotypes may be related to the activities of antifungal proteins (references in Brown et al. 1999). Wounding is performed to bypass the pericarp barrier and provide the fungus with direct access to the kernel internal tissues. In previous studies that investigated *A. flavus* infection of these same corn lines (Brown et al. 1995), wounding increased infection in GT-MAS:gk, but not in MI82. In the present study, wounding increased *F. verticillioides* infection of MI82 kernels, but did not affect infection levels in GT-MAS:gk kernels. Further investigation is needed to determine whether these contrasting responses to fungal species are related to differences in fungal physiology, corn physiology or interactions between the fungus and the host corn. The effect of wounding and/or infection on constitutive levels of TI in kernels of each genotype may also be worth investigating.

Since *F. verticillioides* commonly colonizes corn kernels throughout the world and has been isolated from kernels infected with *A. flavus* (Lillehoj and Zuber 1988), interactions between these two fungi have been studied, but with conflicting results. However, simultaneous inhibition of these two species by TI has been demonstrated in vitro (Chen et al. 1999). These results, along with those highlighted in the present study, provide evidence that corn germplasm may be developed that contains resistance to both aflatoxigenic and fumonisin-producing fungi. Achieving this goal would require that several years of field trials be conducted to assess fungal growth and aflatoxin, as well as fumonisin levels

under varied environmental conditions. This could determine whether a general antifungal resistance is expressed in corn genotypes.

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